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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
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Saswati CHATTERJEE, et al.	)	
	)	
Serial No. 09/453,801	)	Examiner: G. Leffers Jr.
	)	
Filed: December 3, 1999	)	Group Art Unit: 1636
	)	

For: METHOD OF GENETICALLY MODIFYING VERY PRIMITIVE QUIESCENT HUMAN HEMATOPOIETIC STEM CELLS)

DECLARATION UNDER 37 C.F.R. §1.132

Dear Sir:

I, Saswati Chatterjee, do solemnly declare that:

1. I am the same Saswati Chatterjee listed as an inventor on the above-referenced patent application.

2. I received a B.Sc. Degree in biology from McGill University, Montreal Canada in 1976, a M.Sc. Degree in anatomy from McGill University in 1978 and a Ph.D. in the Department of Anatomy of McGill University in 1982, studying the immunobiology of feto-maternal relationships. I did post-doctoral training at the National Institutes of Health, Bethesda, Maryland, in the Immunology Branch, the Laboratory of Genetics of the National Cancer Institute and the Laboratory of Viral Diseases. In 1990, I became an Assistant Professor and Visiting Scientist in the Department of

Microbiology at Georgetown University School of Medicine. In December 1991 I began work as an Assistant Research Scientist in the city of Hope National Medical Center and Department of Molecular Genetics at the Beckman Research Institute of the City of Hope, Duarte, California. I have since been promoted to the position of Associate Research Scientist there and have been studying the use of AAV vectors for stem cell gene therapy and the biology of AAV vectors for approximately four years. A copy of my curriculum vitae is attached herewith as Exhibit 1.

3. I have reviewed and am familiar with U.S. Patent Application Serial No. 09/453,801, filed December 3, 1999, Entitled "A Method of Genetically Modifying Very Primitive Quiescent Human Hematopoietic Stem Cells", including the claims currently pending in the application. I also have reviewed and am familiar with the Office Action of July 18, 2001, and the references cited therein.

4. I, along with Kamehameha K. Wong Jr. and others am an author of the Fisher-Adams et al. reference. The reference describes transduction of cells semi-purified using anti-$CD34^+$ antibodies. These cells are not a population of true stem cells as were the cells

transduced in the present invention. This fact was recognized at the time this paper was published. In addition, the method used to isolate the $CD34^+$ cells was not the same method used and described in the present application.

5. The July 18, 2001 Office Action states that the method of *initially* selecting the $CD34^+$ cell population described by Fisher et al. is essentially the same means applied in the present application and that the media for culture and transduction also are the same. From this, the Office Action concludes that the skilled person would necessarily expect a sub-population of quiescent $CD34^+$ cells residing in G0 and a sub-population of $CD34^{+++}CD38^-$ cells would be present in the cells used for transduction, and that these cells would remain quiescent during the transduction. The factual assertions contained in the Office Action are not correct, and in my opinion the conclusion that a person skilled in the art would expect that quiescent, true stem cells residing in G0 would be transduced by the methods of Fisher-Adams et al. is unjustified. A person knowledgeable about methods for transducing and attempts to transduce true hematopoietic stem cells at the time of Fisher-Adams et al. and before the filing

of the present application would not expect that these cells would have been stably transduced.

6. The methods of Fisher-Adams et al. involved incubation with a mouse anti-CD34 antibody, followed by panning on sheep anti-mouse Ig-coated flasks. To obtain the cells used in the present invention, cells were selected using Multneyi columns followed by appropriate live staining and flow sorting of $CD34^{+++}CD38^{-}/CD34^{+++}$ G0 cells. These cells were sorted based on DNA and RNA content to segregate out only those $CD34^{+}$ cells which were in the G0 phase (see specification, p 17). These G0 cells then were further examined for CD34 and CD38 status and sorted to remove $CD38^{+}$ staining cells. This three-step procedure results in a highly purified population of G0 cells from which G1 cells have been removed. These cells are different, therefore, from any cells which have been transduced in the prior art or in Fisher-Adams et al.

7. The G0 cell cycle status of these purified $CD34^{+++}CD38^{-}$ cells was confirmed a second time based on DNA and RNA content (see specification, p 18, line 23; p 19, lines 1-3 and Tables 1 and 4). The quiescent, non-cycling nature of the cells used also was confirmed using lipophilic membrane dyes, further demonstrating their

G0 status. Our methods resulted in isolation of 0.06% of the total marrow cells, or 6 in 10,000 cells. The frequency of true stem cells is estimated to be between 1 in 10,000 and 1 in 100,000 cells. Thus, we have approached the limit of purification. In my opinion, the evidence, clearly spelled out in the specification of the present application as filed, is more than sufficient to differentiate the cells used by Applicants with the cells of Fisher-Adams et al.

8. The cells transduced by Fisher-Adams et al. were semi-purified $CD34^+$ cells of undetermined cell cycle status. There is nothing in Fisher-Adams et al. to indicate that quiescent, non-cycling pluripotent true stem cells were transduced by the methods used, nor did the authors of Fisher-Adams et al. claim stem cell transduction. The specification, page 10, lines 5-14, discusses this in reference to the disclosures of Fisher-Adams et al. In contrast, the cells transduced and the methods claimed in the present application, require G0 cell cycle status.

9. In addition, the CD34+ cells of Fisher-Adams et al. were cultured in media with cytokines (IL-3, 10ng/ml; IL-6, 5ng/ml; and GM-CSF, 1ng/ml) which are recognized <u>not to support</u> stem cells, since no stem cell factor

was present. Thus, hematopoietic stem cells could not be transduced under these conditions. In contrast, our methods included 1ng/ml Stem Cell Factor (SCF; see Example 3). Under these conditions stem cell survival is expected and was observed. Thus, our culture conditions do allow transduction of stem cells while those used by Fisher-Adams et al. do not.

10. Prior to the invention claimed here, transduction of extremely primitive, G0, quiescent, pluripotent stem cells had not been demonstrated. It is my opinion that this invention therefore is novel and nonobvious; nothing cited in the Office Action from Fisher-Adams et al. teaches anything to the contrary.

11. The Zhou et al. reference is cited as teaching methods of gene transfer into subsets of hematopoietic progenitor cells using AAV-2 vectors, the cells used for gene transfer being $CD34^+$ cells selected by binding to a CD34-specific antibody, *similarly to* those used in Fisher-Adams et al. Zhou et al. identified DNA fragments in various clones obtained from their $CD34^+$ cells after transduction. These progeny clones necessarily had been through one or more cell cycle, therefore the cells could not have been non-cycling. The authors were only able to conclude that their

results "*suggest* that relatively slow *or* noncycling hematopoietic progenitor cell populations in cord blood are susceptible to infection" by AAV, p 1872, col. 1, lines 33-36 (emphasis added). The authors do not claim that they transduced true stem cells. In fact, it is extremely unlikely that the cells transduced by Zhou et al. could have been quiescent cells residing in G0, given the transfection conditions. Zhou et al. used what the Office characterizes, on page 5, line 21, as "low levels" of cytokines (100 U/ml Epo, 100 U/ml IL-3, 100 U/ml GM-CSF). The levels, however, are 10-fold higher than those used in the present invention. As explained in the specification, page 14, lines 9-23, these higher cytokine levels result in mitosis (loss of G0 cell cycle status). It is my opinion that contrary to the conclusion stated in the Office Action, one of skill in the art would *not* expect that the population of $CD34^+$ cells described as transduced by Zhou et al. are quiescent and in G0 cell cycle status.

12. Furthermore, Zhou et al. transduced CFU-C in a short-term (14 day) methyl cellulose colony forming assay. It is well recognized that these cells do not represent either primitive or quiescent cells. These cells have been shown unequivocally to represent lineage committed

cells well into the differentiation process. $CD34^{+++}CD38^{-}$ cells residing in G0 are very primitive and do not readily give rise to colonies in a 14 day CFU-C assay. Thus, a skilled artisan would have concluded that Zhou et al. demonstrated rAAV transduction of cycling, lineage committed, differentiating cells and nothing more. The present invention, on the other hand, has shown transduction of extremely primitive hematopoietic cells in the G0 phase. It is my opinion that the cells claimed in the present application therefore have a novel and unobvious difference from those in the Zhou et al. reference.

13. The Office Action states the *initial* selection of $CD34^{+}$ cells described by Luhovy et al. is essentially the same as that used in the present application. However, Luhovy et al. used methods very similar to those of Zhou et al. They transferred LacZ sequences into $CD34^{+}$ $Lin^{-}Thy^{-}$ cells from presumably adult bone marrow donors. None of the methods used by Luhovy et al. were designed to ensure the G0 cell cycle status of the cells: the antibody-purified $CD34^{+}$ cells were treated only to remove certain specific fully differentiated cell types (see p 25, col. 1). These cells therefore were not the G0-residing cells used in the present

invention as described above and in the specification as filed.

14. The LTC-IC assay employed by Luhovy et al. suggests that the population of cells they used contained more primitive cells than those used by Zhou et al., however the bulk of evidence indicates that cells from LTC-IC assays are still lineage committed and do not represent true stem cells. The fact that retrovirus vectors readily transduce this population indicates that these cells are, in fact, not stem cells residing in G0. Retrovirus vectors do not transduce true stem cells. Therefore, the conclusion that the Luhovy et al. methods transduced cells in G0 cell cycle status, Office Action, page 8, lines 12-19, is completely unwarranted.

15. It is my opinion that the cells claimed in the present application therefore have a novel and unobvious difference from those of the Luhovy et al. reference. Thus, as for the other references cited, Luhovy et al. do not teach the transduction of G0 cells and can not anticipate the claims of the present application.

16. In summary, none of the references cited by the Office as anticipating teach transduction of G0 cells. The methods used by these authors are not the same as those

used in the present application and lack several steps used therein to purify hematopoietic stem cells that exist in G0. Transduction of cells in G0 was not shown. Expansion of the cells used in the prior art revealed that some cell populations had been transduced and carried the foreign DNA, however these colonies of transduced cells are recognized to result from cycling, at least partially differentiated cells. A careful comparison of the methods described by Fisher-Adams et al., Zhou et al., and Luhovy et al. clearly shows that the transduced cell populations are not equivalent to the cells claimed in the present application and that no prior art reference teaches methods for transducing cells in the G0 phase as is claimed in the present application.

17. As further proof that the cells used in this application are true stem cells, we have demonstrated the cell cycle status and quiescent nature of our cells by two different assays: immunophenotyping and in vivo stem cell assays. These assays show that the cells described and claimed in the present application are able to engraft immune-deficient mice in secondary transplants. This is the only available assay to show that cells are true stem cells.

18. The assay was performed as follows. Purified $CD34^{+++}CD38^{-}$ human cord blood cells in the G0 phase, transduced according to the methods of this invention, were transplanted into sublethally irradiated NOD/SCID mice. Engraftment with the transduced human cells, multilineage differentiation of the cells and the ability of the cells engrafted in the bone marrow to give rise to CFU-C colonies were examined. The presence of rAAV sequences in SCID reporting cells and marrow CFU-C also were analyzed.

19. The results clearly showed engraftment of these highly primitive $CD34^{+++}CD38^{-}$ cells as determined by the frequency of human $CD45^{+}$ cells in the mouse bone marrow and spleen. See Figures 1 and 2 attached hereto as Exhibit 2. Engraftment levels rose with time after transplantation, revealing the primitive nature of the transplanted cells. The data show that these cells can engraft and differentiate in the marrow of NOD/SCID mice for at least 25 weeks after bone marrow transplant. More importantly, the engrafted cells differentiated into different hematopoietic lineages. See Figure 3 attached hereto. $CD14^{+}$ and $CD33^{+}$ myeloid and $CD19^{+}$ B lymphocytes were observed consistently. $CD34^{+}$ progenitor cells also were observed in vivo, even

at 25 weeks after transplantation. See Figure 3 attached hereto. The presence of rAAV sequences in these different repopulating lineages were determined by amplification of the vector sequences and prove that the transplanted and stably transduced cells were true stem cells, capable of multipotential differentiation. See Table 1 attached hereto.

20. The self-renewal capacity of the rAAV transduced cells and their progeny was determined by plating the cells in very long term colony forming assays. CFU-GEMM (colony forming unit -- granulocyte, erythrocyte, monocyte, macrophage) colonies were obtained from the transduced cells, again showing that the transduced cells were highly primitive, multipotential cells. See Figure 4 attached hereto.

21. Bone marrow cells were harvested from the primary hosts approximately 7-8 weeks after bone marrow transplant and infused into secondary sublethally irradiated recipients. Analysis of these secondary recipients revealed the presence of rAAV sequences in the myeloid and lymploid cells in the bone marrow and spleen of 4 out of 4 animals, indicating that self-renewing *true stem cells* capable of extended survival had been transduced in the original innoculum. See Figure 5.

Importantly, rAAV sequences were detected in the $CD34^+$ population in the bone marrow of a secondary recipient, indicating that the methods claimed in the present application were able to transduce *true stem cells* using rAAV.

22. These new data conclusively demonstrate the stable transduction of the highly primitive, quiescent stem cells residing in G0. In my opinion this is more than sufficient to show the novel and nonobvious differences between the product claimed in this application and those of the cited prior art references.

23. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may

jeopardize the validity of the application or any patent issued thereon.

Saswati Chatterjee

January 3, 2003
Date

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